

ABSTRACT

CHEMISTRY

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PHOTOCATALYTIC INACTIVATION OF FUNGI WITH TiO₂ WITH WHITE LIGHT AND DIFFERENT BUFFER SYSTEMS

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The photocatalytic inactivation of fungi with P25 a mixed phase TiO₂ material (25 % rutile and 75 % anatase) was examined using four fungal species: *A. niger* and *M. racemosus*, both spore forming fungi, and *C. albicans* and *S. cerevisiae*, yeast forming fungi. All four fungi species were found to be highly resistant to photocatalytic inactivation with P25 at room temperature under warm white light.

The photocatalytic inactivation of fungi with P25 and alumina, in the presence of bicarbonate, chloride, phosphate, and silver were studied under warm white light to determine if these additives could enhance the inactivation activity. The addition of chloride and phosphate did not improve the inactivation of the fungi. Alumina lead to slight improvement in photocatalytic inactivation and silver/P25 inactivated the fungi even in the dark. The addition of bicarbonate, which is found in natural waters, dramatically increased the photocatalytic inactivation activity of the P25. The photocatalytic inactivation activity of P25 in bicarbonate was found to be pH dependent, with activity increasing with decreasing pH. However, the pH cannot be reduced below 6, because of H₂CO₃ formation followed by its decomposition to CO₂ and water. The rate of photocatalytic inactivation *C. albicans* with P25 and bicarbonate

was studied at pH 6.06 and 10. The morphology of *C. albicans* was examined microscopically at 60x. Upon photocatalytic inactivation in the presence of bicarbonate it was observed that the cells were totally fragmented and it appeared that most of the cytoplasm had leaked out.

PHOTOCATALYTIC INACTIVATION OF FUNGI WITH TiO_2 UNDER WHITE
LIGHT AND DIFFERENT BUFFER SYSTEMS

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LIST OF ABBREVIATIONS

TiO ₂	Titanium Dioxide
AIDS	Acquired immunodeficiency syndrome
UV	Ultraviolet
UVA	Ultraviolet A rays
ROS	Reactive Oxygen Species
PBN	Phenyl-t-butyl nitron
POBN	4-pyridyl-1-oxide-N-t-butyl-nitron
DMPO	5,5-dimethyl-1-pyrroline N-oxide
EPR	Electron paramagnetic resonance
2,4 DNPH	2,4 Dinitrophenylhydrazine
YPG	Yeast peptone glycerol
YPD	Yeast peptone dextrose or glucose
YEPD	Yeast extract peptone dextrose

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

TiO₂ has been widely studied as a photocatalyst for the destruction of organic materials in water, and more recently as a photodisinfection catalyst.¹ Considerable experimental evidence for the biocidal efficacy of TiO₂ photocatalysis has been found in recent years; however, the mechanism of disinfection of bacteria and viruses is still an area of active study.²⁻⁷ Previous research in our group has shown that the efficiency of photocatalytic inactivation of *Escherichia coli* K12 and bacteriophage MS-2 with titanium dioxide (TiO₂) varies with pH, salts and buffers present in solution. This led us to examine the variation, if any, in the photocatalytic inactivation of fungi as a function of pH and the presence of aqueous salts and buffers.

1.0 Life Cycle of *Candida albicans*

The genus *Candida* belongs to the *saccharomycetacea* family and is described as a white imperfect yeast capable of forming pseudohyphae. The genus *Candida*, species are characterized primarily on colonial morphology, carbohydrate utilization, and fermentation.⁸ Yeast are Gram positive and grow overnight on fungal media. *Candida*

has the ability to switch between growing as unicellular yeast or as multicellular filaments.⁹ The cell wall of the fungi is rigid and contains soluble and insoluble polysaccharide polymers, like chitin, β -glucans and glycoproteins. *C. albicans* is 10-12 microns in diameter, which is 25-50 times bigger than most bacteria.¹⁰

C. albicans, as a nonphotosynthetic microorganism, needs carbon and nitrogen sources as well as some growth factors such as biotin for their growth. Rather than division through binary fission, they divide by budding.¹¹ *C. albicans* is a dimorphic microorganism that has the ability to grow in two different ways. The first way is reproduction by budding, forming an ellipsoid bud, and in the hyphal form, which periodically fragments and give rise to new mycelia or yeast like forms.¹¹ The budding phenotype is common at low temperatures or low pH; however, the hyphal phenotype is common at high temperatures and high pH. Germ tubes and buds are formed on the surface of blastospores, which is the unicellular part of the yeast. Hypha are formed when the germ tubes grow and their septa are laid down behind the extended apical tip. Mycelium are then produced when the secondary branches or hyphal branches are produced behind the laid down septa. Secondary blastospores then separate from their filaments.¹¹

1.1 Pathogenicity of *Candida albicans*

C. albicans causes infections in cancer, AIDS, and other immunosuppressed patients. Candidiasis (yeast infection) may be life threatening as it adheres to the host tissue, which then produce damaging enzymes. In 15-30% of healthy people *C. albicans* lives in the gastrointestinal or genital tract.¹² *C. albicans* is the causative agent of vaginal

discharge, oral candidiasis, and life threatening invasive infections. *C. albicans* is also the leading cause of nosocomial infections. Nosocomial is common in 60% of candidemia patients.¹² Thrush is also a common infection that's found in the mouth and throat of infants. Thrush occurs as white lesions on the mucus membrane of the mouth and tongue. Woman who take antibiotics and steroids over a long period of time often catch the infection and transfer it to their infants at birth. Vaginal infections caused by *C. albicans* are common in women. The yeast infection leads to a thick whitish vaginal discharge that may cause discomfort and itch. Men can also have irritation on their genitals caused by *C. albicans*. Vulvitis is a known fungal disease that occurs on the vulva also caused by *C. albicans*, which are most frequent in diabetic patients.¹³ Itraconazole and fluconazole are the drugs of choice for the treatment candidiasis.^{14,15}

1.2 Life Cycle of *Aspergillus niger*

A. niger, a black mold found on plants, such as fruits and vegetables, is known for its production of citric acid, and can be detrimental to humans if inhaled.¹⁶ Its genome is estimated to be between 35.5 and 38.5 megabases.¹⁷ *A. niger* plays a significant role in the carbon cycle because it is a soil saprobe with a wide array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocellulose.¹⁷

1.3 Life Cycle of *Mucor racemosus*

The genus *Mucor* belongs to the *mucoraceae* family and is a fungal pathogen. *M. racemosus* is dimorphic exhibiting two vegetative cell types; budding yeasts and branched hyphae that are produced during the cell cycle.¹⁸ *M. racemosus* is a saprophyte

and is detrimental to fruits and vegetables. *M. racemosus* can grow into two different forms, yeast and mold. Mold or filamentous growth is induced by air or nitrogen, which causes the hyphae to break up into spherical cells called arthrospores.¹⁹ If the environment is anaerobic and CO₂ is introduced *M. racemosus* to form spherical budding producing yeast-like cells.¹⁹ The yeast forms under aerobic conditions when induced with CO₂ or N₂. The mold form consists of non-septate hyphae with specialized cells for asexual and sexual reproduction.²⁰

1.4 Life Cycle of *Saccharomyces cerevisiae* (Baker's Yeast)

The genus *Saccharomyces* belongs to the *Saccharomycetaceae* family and forms round ovoid cells 5-10 micrometers in diameter. *S. cerevisiae* divides by budding with a small bud emerging from the surface of the parent cell that enlarges until it is almost the size of the parent cell. Concurrently the chromosomes of the parent cell replicate.²¹ At mitosis, when the nucleus divides, one of the nuclei is transferred to the bud, and two cells separate.²¹

1.5 Titanium Dioxide (TiO₂) Photocatalysis

Titanium dioxide is a photocatalyst that has been used for environmental restoration, because of its high oxidative activity under photocatalytic conditions, is insoluble in water, and non-toxic.²²⁻²⁵ TiO₂ has three crystal forms: rutile, anatase, and brookite. At an energy level higher than its band gap, titanium dioxide absorbs light causing electrons to be promoted to the conduction band producing positive holes in the valence band.²⁶ Degussa P25 is a mixed phase TiO₂ (25 % rutile and 75 % anatase) is

widely used for photocatalysis. The band gaps of rutile and anatase are 3.0 and 3.2 eV, respectively. Anatase exhibits a higher level of photocatalytic activity than rutile because of the difference in the position of the conduction bands. When titanium oxide absorbs light, electrons (e^-) and positive holes (h^+) are formed inside the crystals that can migrate to the surface of the crystal. Electrons and positive holes recombine slowly in titanium oxide photocatalyst in comparison to other semiconductors. Upon UV light photolysis in water, in the presence of O_2 , TiO_2 photocatalyst generate reactive oxygen species (ROS) including OH , H_2O_2 , and $O_2^{\cdot-}$ which can damage microorganisms.^{26,27} The positive hole can oxidize water at the surface, forming hydroxy radicals ($\cdot OH$) with strong oxidative decomposing power, which react with organic matter. In the presence of oxygen, the intermediate organic radicals and undergo radical chain reactions consuming oxygen. In some cases, the organic matter is decomposed to carbon dioxide and water. The OH radical is very toxic to microorganisms because it has the ability to oxidize carbohydrates, lipids, proteins, and nucleic acids.²⁸ Peroxidation of polyunsaturated phospholipid components of the lipid membrane promoted by OH radicals induce disorder in the cell membrane.²⁹ When organic compounds react directly with the positive holes, oxidative decomposition occurs.²⁶ Superoxide anions ($O_2^{\cdot-}$) are also formed by the reduction of oxygen.

1.6 Photocatalytic Antifungal Activity of TiO_2

It was reported by Dariusz Mitoraj *et al.* that the inactivation of *C. albicans* was achieved under UV light in the absence of TiO_2 .³⁰ *A. niger* was reported to be more resistant to UV light than *C. albicans*.³⁰ Maneerat and Hayata *et al.* studied the

photocatalytic antifungal activity of TiO₂ against *Penicillium expansum* *in-vitro* and on tomatoes.³¹ They found that the fruit, when treated with TiO₂ alone or UVA ($\lambda = 320$ -400 nm) alone did not inactivate *P. expansum*. However, when they illuminated tomatoes treated with TiO₂ under UVA light, the number of the *P. expansum* was reduced significantly. The rate of spore reduction correlated with the amount of TiO₂ used. Hur *et al.* investigated the effects of TiO₂ photocatalytic oxidation and ozonation on the control of postharvest fungal spoilage of kiwi fruit.³² They found that ozonation was more effective at inhibiting conidial germination than TiO₂ under photocatalytic conditions. They also documented that co-treatment with ozone and photocatalysis with TiO₂ completely inhibited the fungal spoilage of kiwi fruit and demonstrated a higher fungicidal activity than flusilazole.³² In another study, Chen *et al.* tested the antifungal capability of a TiO₂ film on moist wood.³³ Their findings suggest that TiO₂ does not act as a germicide under ambient indoor lighting. However, UVA illumination enhanced the TiO₂ photocatalytic disinfection processes and was effective for the inhibition of *A. niger* growth.³³ They also reported that the UVA light photocatalytic disinfection processes was effective for inhibition of spore germination of mold fungi (*A. niger*).

1.7 Oxidative Stress on Fungi

Oxidative stress is defined as a disturbance in cell or organism pro-oxidant-antioxidant balance in favor of the former.³⁴ Hyperbaric oxygen, γ -radiation, near-UV radiation, ozone, peroxides and redox-cycling drugs are external oxidative stresses that have proven detrimental for both eukaryotic and prokaryotic cells.³⁵ Oxidative stress is very different from other stresses on microorganisms because of its primary effectors, the

reactive oxygen species (ROS), can arise in the course of normal cell metabolism.³⁶

Ames and coworkers stated: *“In the course of O_2 reduction to $2 H_2O$ and oxidation of organic substances to CO_2 during the cell energy-yielding reactions up to 2-3% of the oxygen molecules are reduced only partially, giving rise to different reactive oxygen species.”*³⁷ Superoxide radicals, hydrogen peroxide, and hydroxy radicals are the primary oxidants that form as by-products of energetic metabolism in cells. *“All these primary oxidative stressors can generate additional secondary reactive oxygen metabolites which also cause extensive oxidative damage to cell organelles, such as mitochondria, cell membranes or nuclei, and also to both soluble and bound enzymes.”*³⁷⁻³⁹

Angelova *et al.* studied oxidative stress on fungi induced by hydrogen peroxide (H_2O_2) and paraquat (PQ).⁴⁰ When spores were exposed to PQ and H_2O_2 there was a reduction in spore germination, with H_2O_2 giving a higher reduction than PQ. When the effect of PQ and H_2O_2 were tested on fungal growth, they both decreased the production fungal biomass. The fungal biomass was 3-4 times lower when fungi were treated with H_2O_2 than when untreated.⁴⁰ Protein oxidation occurring in fungi during oxidative stress was also examined by Angelova *et al.*⁴⁰ They used the reaction of 2,4-dinitrophenylhydrazine (DNPH) to determine the carbonyl content of the proteins in the fungi treated with PQ or H_2O_2 .⁴⁰

Cabiscol *et al.* studied the oxidative stress that promotes specific protein damage in *S. cerevisiae*.⁴¹ They treated *S. cerevisiae* with hydrogen peroxide in YPG (1% yeast extract, 2% peptone, 3% glycerol) and YPD (1% yeast extract, 2% peptone, 2% glucose). YPG medium uses glycerol as its carbon source and YPD uses glucose as its carbon source. The basal levels of the protein carbonyl content was higher in the cells that were

grown in YPG, however there was also an increase in protein damage in the cells that were grown in YPD.

CHAPTER 2

EXPERIMENTAL SECTION

2.0 Materials

Candida albicans H317 was donated by Dr. David Logan in the department of biological sciences at Clark Atlanta University. *Aspergillus niger* (ATCC® 16404™) and *Mucor racemosus f. lusitanicus* (ATCC® 1216B™) were purchased from ATCC. *Saccharomyces cerevisiae* (the Fleishmanns strain) was purchased from a local grocery store. Yeast extract peptone dextrose (YPD) broth/agar, potato dextrose agar (PDA) broth/agar, yeast extract bacto peptone glucose (YPG) broth/agar, sodium bicarbonate buffer, phosphate buffer, phosphoric acid, NaCl, 2,4-dinitrophenylhydrazine (2,4-DNPH), HCl, and NaOH, were purchased from Fischer Scientific. DISPAL® 23N4-80 alumina powder was purchased from Sasol. Calf serum was purchased from BIOfluids. Degussa P25 TiO₂ was purchased from Degussa.

2.1 Fungal Culture and Media Preparation

The growth medium, yeast extract peptone dextrose (YPD), was prepared from 10.0 g of Bacto™ yeast extract, 20.0 g of Bacto™ tryptone, and 20.0 g of dextrose (D-glucose) in 1.00 L of distilled water. YPD agar was prepared in a similar manner from

20.0 g of agar in 1.00 L of distilled deionized water (DDI H₂O). The mixture was then boiled to dissolve the agar and then autoclaved at 121°C and 18 psi for 45 to min.

2.2 Preparation of Salt and Buffer Solutions

A 100 mM sodium bicarbonate buffer was prepared from 4.2 g of sodium bicarbonate and 500 mL of autoclaved water. The pH of this solution was adjusted as required to 6.00, 8.00, and 10.00 using either HCl, acetic acid, or NaOH as needed. The pHs of phosphate buffers was adjusted to 4.47, 6.06, 7.07, and 8.42 using concentrated phosphoric acid or NaOH as needed. A 150 mM NaCl solution was prepared with a pH of 6.7. The pH of water was adjusted using either HCl or acetic acid as needed.

2.3 Plating and Harvesting *Candida*, *Saccharomyces*, *Mucor*, and *Aspergillus* Cells

C. albicans and *S. cerevisiae* were grown in YPD broth and incubated overnight at 30° C. The culture was then stored in 20 % glycerol in a -20° C freezer and used as a stock culture. *M. racemosus* was grown in YPG broth and *A. niger* was grown in PDA broth for 2-3 d. *C. albicans* and *S. cerevisiae* stock cultures were revived in YPD broth overnight and used as a source of the working culture. A 1.5 mL sample of the working culture was placed in 25.0 mL of YPD agar in a 50 mL culture tube. The culture tubes were placed in an incubator at 30° C for 6 h, and then centrifuged at 4,000 rpm for 7 min to form a pellet of cells at the bottom of the tube. The *C. albicans* and *S. cerevisiae* pellets were washed twice with autoclaved distilled deionized H₂O and then resuspended in autoclaved distilled deionized water to give suspensions of 1.0×10^6 to 1.0×10^7

cells/mL. In some cases a hemacytometer grid (Hausser Scientific; Horsham, PA) was used under a compound light microscope (MicroMaster Fisher Scientific; Pittsburgh, PA) to determine the final cell concentrations. In other cases the number of the spores in the suspension was estimated by measuring the optical density at 495 nm using a Turner Spectrophotometer Model 350, and comparing the optical density with a standard curve.

To obtain the optimum wavelength for estimating cell count, a suspension of *A. niger* spores in water was scanned using a Turner Model 350 spectrophotometer. The optical density (O.D.) at 495 nm was chosen to develop a calibration curve for the estimation of *A. niger*, (Figure 1). The calibration curve was constructed by plotting the O.D. at 495 nm vs. the plate count for the same suspensions.

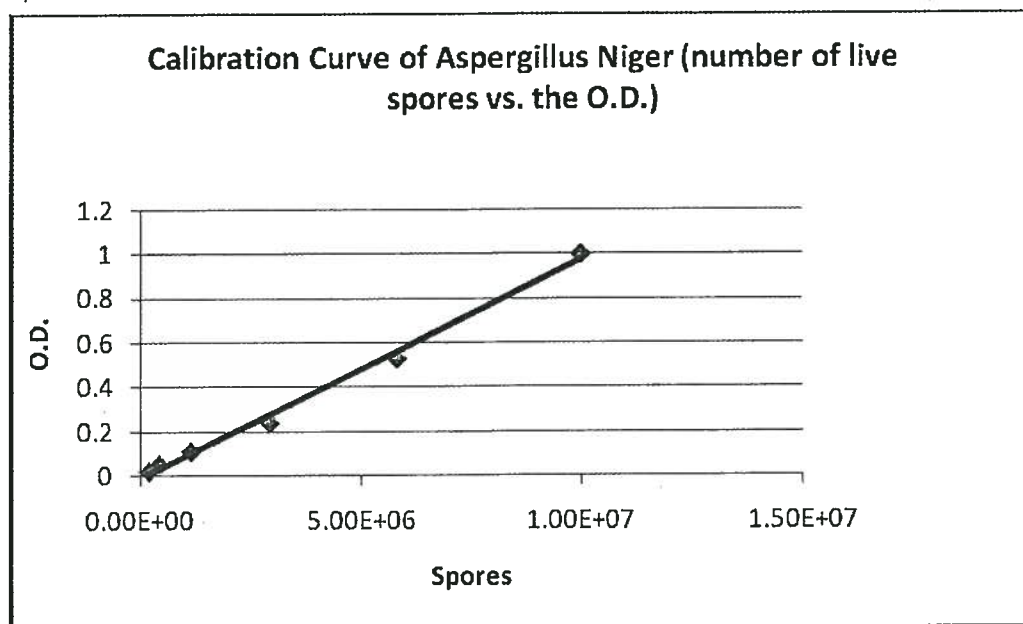


Figure 1. Number of live *A. niger* Spores vs. Optical Density

M. racemosus was spread over YPG agar and grown for 2 d. *A. niger* was spread over PDA agar and grown for 3-4 d. The spores were then collected and diluted to a final

concentration between 1.0×10^4 and 1.0×10^6 spores/mL. The optical density method was used to estimate the number of the spores in the fungal suspension.⁴²

2.4 Photocatalytic Antifungal Activity of TiO_2

The photocatalytic treatment of water suspensions of *C. albicans* were carried out with 0.5 or 1.0 mg of TiO_2 , Al_2O_3 , $\text{TiO}_2/\text{Al}_2\text{O}_3$, or $\text{TiO}_2/\text{Al}_2\text{O}_3/\text{HIO}_3$ composite per mL in sterile polystyrene petri dishes. A series of experiments was carried out with TiO_2 suspended in water, salts and buffers at various pHs. The experiments were carried out in duplicate and conducted under warm white light (8 W Hitachi F8T5) or in the dark. For tests in the dark, the petri dishes were wrapped with aluminium foil. The samples were then placed on a rotator (Fisher rotator 14-251-200) for 3 and 24 h at 80 rpm agitation. After the desired time, samples from each petri dish were serially diluted. The direct, 10^2 , 10^4 , 10^6 dilutions were then plated on YPD agar plates and incubated at 30°C for 2 d and the colonies counted.

2.5 Germ Tube Formation

Using a sterile loop, colonies from previously tested *C. albicans* plates were aseptically suspended in 1.0 mL of calf serum donor in a 15 mL disposable polypropylene centrifuge tube. The resulting mixtures were incubated at 37°C for 2-4 h. After the desired time, 10.0 μL of the yeast-serum mixture was pipetted on to a clean microscope slide, covered with a cover slip and examined microscopically, using a 60x objective lens. The appearance of tubes branching off of the yeast was then observed and photographed.

2.6 Treatment of Photocatalytically Treated Fungi with 2,4-Dinitrophenylhydrazine (2,4-DNPH)

A 0.1 % (w/v) solution of 2,4-DNPH was prepared with 2 M HCl in a 100 mL beaker. Samples (15 mL) from the *C. albicans* inactivation experiments described in 2.4 above were pipetted into 15 mL sterile disposable polypropylene centrifuge tubes. The tubes were then centrifuged at 4,000 rpm for 7 min to give a pellet of cells and cell debris at the bottom of the tube. The supernatant was then poured off the pellet into a separate 15 mL sterile polypropylene disposable centrifuge tube followed by 2.0 mL of the 2,4-DNPH reagent and incubated for 30 min at 30° C. After incubation, 2.0 mL of 1 M NaOH was added and the sample was allowed to stand at room temperature for 5 min. The absorbance of the mixtures was then measured at 427 nm with a Turner Model 350 spectrophotometer.

2.7 Study of the Morphology Changes of *C. albicans* after Treatment with TiO₂

A smear was prepared by aseptically transferring fungal samples to microscope slides. The slides were air dried and then passed three times through a Bunsen burner flame to heat-fix and kill the fungi. The slides were then stained with crystal violet for 30 sec. The excess stain was then washed off with water and the slide blotted dry with a paper towel. The slides were examined under a compound light microscope (MicroMaster Fisher Scientific; Pittsburgh, PA), and the morphology determined visually and photographed.

2.8 Antifungal Photocatalytic Activity of TiO₂, Ag doped TiO₂ and Ag/Al/TiO₂ Composites on *A. niger*

The photocatalytic treatment of water suspensions of *A. niger* was carried out with 0.1 or 1.0 mg/mL suspensions of TiO₂, Ag/TiO₂, or silver doped TiO₂/Al₂O₃ in sterile polystyrene petri dishes. Three samples of silver doped TiO₂/Al₂O₃ were examined with silver doping levels of 591., 906., and 1,540. ppm, respectively. The experiments were carried out in duplicate and conducted under warm white light (8 W Hitachi F8T5) and in the dark. For the tests and controls in the dark, the petri dishes were wrapped with aluminium foil. The samples were then placed on a rotator (Fisher rotator 14-251-200) for 2, and 72 h at 80 rpm agitation. After the desired time, samples from petri dishes were serially diluted. The direct, 10², 10⁴, 10⁶ dilutions were then plated on PDA plates and incubated at 30° C for 4 d and the colonies counted.

2.9 Antifungal Photocatalytic Activity of TiO₂/Al₂O₃/HIO₃, Al₂O₃/TiO₂ mix, Al₂O₃, and TiO₂ on *M. racemosus*

The photocatalytic treatment of water suspensions of *M. racemosus* were carried out by treating suspensions of *M. racemosus* with 0.05, 0.10, or 1.0 mg of TiO₂/Al₂O₃/HIO₃, Al₂O₃/TiO₂ mix, Al₂O₃, or TiO₂ per mL in sterile polystyrene petri dishes. The experiments were carried out in duplicate and conducted under warm white light (8 W Hitachi F8T5) and in the dark. For the tests and controls in the dark, the petri dishes were wrapped with aluminium foil. The samples were then placed on a rotator (Fisher rotator 14-251-200) for 1 and 3 h at 80 rpm agitation for the TiO₂ and Al₂O₃/TiO₂ mix tests. However, the TiO₂, Al₂O₃/TiO₂ mixture, and Al₂O₃ were treated for 6 and 24 h and the TiO₂, Al₂O₃/TiO₂ mix, Al₂O₃, and TiO₂/Al₂O₃/HIO₃ were tested for 3 h. After

the desired time, samples from the petri dishes were serial diluted. The direct, 10^2 , 10^4 , and 10^6 dilutions were then plated on YPG plates and incubated at 30 °C for 2 d and the colonies counted.

2.10 Antifungal Photocatalytic Activity of $\text{TiO}_2/\text{Al}_2\text{O}_3/\text{HIO}_3$, $\text{Al}_2\text{O}_3/\text{TiO}_2$ mix, Al_2O_3 , and TiO_2 on *S. cerevisiae*

The photocatalytic treatment of water suspensions of *S. cerevisiae* were carried out by treating suspensions of *S. cerevisiae* with 0.005, 0.025, or 0.05 mg/mL of $\text{TiO}_2/\text{Al}_2\text{O}_3/\text{HIO}_3$, $\text{Al}_2\text{O}_3/\text{TiO}_2$ mixtures, Al_2O_3 , or TiO_2 in a sterile polystyrene petri dish. The experiments were run in duplicate and conducted under warm white light (8 W Hitachi F8T5) and in the dark. For the tests in the dark, the petri dishes were wrapped with aluminium foil. The samples were then placed on a rotator (Fisher rotator 14-251-200) for 1 and 3 h at 80 rpm agitation for the TiO_2 , $\text{Al}_2\text{O}_3/\text{TiO}_2$ mixture, and the Al_2O_3 test were tested for 6 and 24 h and the $\text{TiO}_2/\text{Al}_2\text{O}_3/\text{HIO}_3$ composite tests was conducted for 3 and 6 h. After the desired time, samples from each petri dish were serial diluted. The direct, 10^2 , 10^4 , 10^6 dilutions were then plated on YPD plates and incubated at 30° C for 2 d and the colonies counted.

CHAPTER 3

RESULTS AND DISCUSSION

3.0 Photocatalytic Antifungal Activity of TiO₂

Titanium dioxide, TiO₂, has previously been studied for the photocatalytic inactivation of bacteria and viruses.⁴³ A few researchers have reported that TiO₂ exhibits limited photocatalytic antifungal activity.³¹ However, we decided, based on other work carried out in our laboratory, to determine if the photocatalytic activity of TiO₂ against fungi could be improved by the use of additives.⁴⁴ To set a baseline and to determine if spore forming and non spore forming fungi respond differently to photocatalytic treatment with TiO₂, we initially examined the activity of four fungal species in water with P25 under warm white light; *A. niger*, *M. racemosus*, *S. cerevisiae* (Baker's Yeast), and *C. albicans*. *A. niger* and *M. racemosus* are spore forming fungi, and *C. albicans* and *S. cerevisiae* are yeast forming fungi.^{9,16,20,21} The cell wall of both forms contain mannoproteins, glycoproteins, β -glucans, β -glucans and chitin.^{45,46} A major difference between the spore forming and non spore forming fungi is the length of the glycoproteins that make up the cell wall. Yeast have short chains glycoproteins and spore forming fungi have longer galactose and mannose containing glycoproteins.^{45,46}

3.1 Photocatalytic Inactivation of *A. niger*, *M. racemosus*, *S. cerevisiae*, and *C. albicans* with P25 in water

A. niger, *M. racemosus*, *S. cerevisiae*, and *C. albicans* were treated with 0.05, 0.1 or 1.0 mg/mL suspensions of P25 in 15.0 mL of fungal suspensions under warm white light and in the dark for varying times. In a manner similar to previous studies³⁰⁻³³ P25 exhibited only limited effectiveness for the inactivation of *A. niger*, even after 72 h of treatment. This treatment in light was only slightly more effective than treatment in the dark, see Table 1. While the inactivation of over 99% of the *A. niger* after 2 h appears impressive, it must be kept in mind that microorganisms, unlike chemicals, are alive and can grow back. Therefore, the benchmark for microorganism inactivation to be considered successful is a five to six log reduction.

Table 1. Antifungal activity of TiO₂ under warm white light

Time (h)	Pure H ₂ O		TiO ₂ (1.0 mg/mL)		TiO ₂ (0.1 mg/mL)	
	D	L	D	L	D	L
0	2.8×10^6	2.8×10^6	2.8×10^6	2.8×10^6	2.8×10^6	2.8×10^6
2	2.0×10^6	2.4×10^6	1×10^3	3×10^3	1×10^3	2.6×10^2
72	1.2×10^6	1.8×10^6	4.8×10^2	8.5×10^2	1.5×10^2	2.1×10^2

Photocatalytic treatment of *M. racemosus*, another spore forming fungi, with 0.05 mg/mL of P25 was not only ineffective, but the *M. racemosus* continued to reproduce under photocatalytic conditions, see table 2. Treatment of *S. cerevisiae* and *C. albicans*, a yeast-forming fungi, with P25 under photocatalytic conditions also proved ineffective, see Table 2.

Table. 2. Photocatalytic inactivation of *M. racemosus*, *S. cerevisiae*, and *C. albicans* under warm white light in water with P25

Time (h)	<i>M. racemosus</i>		<i>S. cerevisiae</i>		<i>C. albicans</i>	
	Control	TiO ₂ (0.05 mg/mL)	Control	TiO ₂ (0.05 mg/mL)	Control	TiO ₂ (0.05 mg/mL)
0	1.65×10^4	1.65×10^4	3.68×10^6	3.68×10^6	1.0×10^5	1.0×10^5
6	5.0×10^4	1.12×10^5	7.77×10^6	6.66×10^6	1.0×10^5	3.4×10^5

3.2 Photocatalytic Inactivation of *Candida albicans* with a Physical Mixture of P25 and Alumina and a TiO₂/Al₂O₃ Composite in Water

Previous studies in our research group have shown that TiO₂/Al₂O₃ composites exhibit higher photocatalytic activity against bacteria and viruses than P25 alone under some conditions.⁴⁷ Therefore, we examined the photocatalytic fungal inactivation activity of P25 physically mixed with alumina powder, Dispersal[®] 23N4-80, and with a TiO₂/Al₂O₃ composite prepared by the hydrolysis of titanium (IV) isopropoxide and aluminum (III) *sec*-butoxide in the presence of HIO₃.⁴⁸ After 24 h, the *C. albicans* continued to reproduce slowly under the photocatalytic conditions with P25. However, the number of viable *C. albicans* was decreased by approximately 99% with alumina alone and 99.9% with a physical mixture of TiO₂ and alumina under the same conditions. The TiO₂/Al₂O₃ composite did not prove to be as effective as the physical mixture of TiO₂ and alumina at inactivating *C. albicans* under photocatalytic conditions, see Table 3. Under similar experimental conditions, *A. Niger*, *M. racemosus*, *S. cerevisiae* and *C. albicans* were found to be resistant to photocatalytic inactivation.

Table 3. Photocatalytic inactivation of *C. albicans* with TiO₂ and TiO₂/Al₂O₃

Time (h)	Control	TiO ₂ (1.0 mg/mL)	Al ₂ O ₃ (1.0 g/mL)	Al ₂ O ₃ (0.5 mg/mL) TiO ₂ (0.5 mg/mL)	TiO ₂ /Al ₂ O ₃ (1.0 mg/mL)
0	1.0 x 10 ⁵	1.0 x 10 ⁵	1.0 x 10 ⁵	1.0 x 10 ⁵	1.0 x 10 ⁵
3	1.0 x 10 ⁵	3.4 x 10 ⁵	1.1 x 10 ⁵	8.2 x 10 ⁴	1.5 x 10 ⁴
24	1.0 x 10 ⁵	6.0 x 10 ⁵	9.0 x 10 ³	7.5 x 10 ²	1.4 x 10 ⁴

3.3 Photocatalytic Inactivation of *A. niger* with Ag Doped TiO₂/Al₂O₃ Composites

Samples of TiO₂/Al₂O₃ doped with 591., 906., and 1,540. ppm of silver were provided by Dr. Liang Liao of our research group. Photocatalytic inactivation of *A. niger* with the silver doped composites in water was monitored for 24 h under warm white light and the dark, see Table 4. The silver doped TiO₂/Al₂O₃ composites were able to produce a five log reduction of the fungi in the first hour under visible light and in the dark. These results suggest that the inactivation due to the silver is far faster than the photocatalytic inactivation due to P25, hence we discontinued this study as it did not allow us to examine the photocatalytic effects even at the lowest silver doping level.

Table 4. Inactivation of *A. niger* with silver doped TiO₂/Al₂O₃ composites under cool white light and in the dark.

Time (h)	TiO ₂ /Al ₂ O ₃ (0.1 mg/mL 591. ppm Ag)		TiO ₂ /Al ₂ O ₃ (0.1 mg/mL 906. ppm Ag)		TiO ₂ /Al ₂ O ₃ (0.1 mg/mL 1,540. ppm Ag)		Water	
	Dark	Light	Dark	Light	Dark	Light	Dark	light
0	5.2 x10 ⁵	5.2x10 ⁵	5.2x10 ⁵	5.2 x10 ⁵	5.2x10 ⁵	5.2x10 ⁵	5.2 x10 ⁵	5.2 x10 ⁵
1	0	4	0	12	0	0	8.4x10 ²	7.8x10 ²
2	0	0	0	0	0	0	7.4x10 ²	8.4x10 ²
24	0	0	0	0	0	0	3.2x10 ²	1.0x10 ³

3.4 The Effect of the pH and Additives on the Photocatalytic Disinfection of *C. albicans* with P25

Previous studies by our research group and others have shown that the photocatalytic inactivation activity of P25 against bacteria and viruses can be altered by the addition of Cl⁻, PO₄³⁻ and HCO₃⁻.⁴⁴ *C. albicans* was treated with 1.0 mg/mL of P25 under cool white light in the presence of Cl⁻, PO₄³⁻ and HCO₃⁻ at various pHs. For these studies 100 mM sodium bicarbonate solutions were prepared and adjusted to pH 6.06, 8.10, and 10.12 using acetic acid or NaOH, 11 mM phosphate solutions were prepared and the pH adjusted to 4.17, 6.08, 8.42 using concentrated phosphoric acid or NaOH, and a 150 mM NaCl solution was prepared at pH of 5.90. The pH of the water was adjusted using acetic acid or NaOH as necessary.

P25 in sodium bicarbonate buffer at a pH of 10.12 exhibited higher photocatalytic disinfection activity against *C. albicans* than the phosphate or chloride solutions studied, (Table 5), resulting in a four log reduction in the number of viable *C. albicans*. In the

phosphate buffer at pH 8.42, NaCl at pH 5.90 and water at pH 7.6 *C. albicans* was decreased by only two logs under the photocatalytic treatment with P25, see Table 5.

Table 5. Photocatalytic inactivation of *C. albicans* with bicarbonate, phosphate and chloride at various pHs under warm white light

Time (h)		Bicarbonate pH 10.12	Phosphate pH 4.17	Phosphate pH 6.08	Phosphate pH 8.42	NaCl pH 5.90	Water pH 7.6
0 h	CFU	8.2×10^7	8.2×10^7	8.2×10^7	8.2×10^7	8.2×10^7	8.2×10^7
	pH	10.2	4.8	6.4	8.8	5.78	7.64
20 h	CFU	3.0×10^3	5.6×10^6	1.1×10^7	1.4×10^5	3.0×10^5	9.8×10^5
	pH	10.2	4.17	6.1	8.48	5.80	8.54

The photocatalytic inactivation of *C. albicans* with P25 in bicarbonate solutions vs. pH was studied at pH 6.06, 8.05, and 10.12. It was observed that as the initial pH of the buffer was decreased the photocatalytic antifungal activity of the P25 increased, see Table 6. Decreasing the pH increases the bicarbonate to carbonate ratio in the solution. In a similar manner the photocatalytic inactivation *C. albicans* with P25 in phosphate solutions vs. pH was studied at pH 4.17, 6.08, 7.1 and 8.42. It can be seen in Table 7 that varying the pH the phosphate buffer did not significantly improve the photocatalytic inactivation of *C. albicans*.

Table 6. Photocatalytic inactivation of *C. albicans* with P25 and bicarbonate at various pHs

	Bicarbonate pH:6.06				Bicarbonate pH:8.10				Bicarbonate pH:10.12			
	Test (TiO ₂)		Water		Test (TiO ₂)		Water		Test (TiO ₂)		Water	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark
Initial pH	6.50	6.48	6.41	6.46	8.44	8.42	8.35	8.32	10.01	10.00	10.02	10.03
Final pH	8.97	8.99	9.04	8.83	9.46	9.32	9.50	9.36	8.87	9.25	8.84	8.69
0 h	4.6x10 ⁶	4.6x10 ⁶	4.6x10 ⁶	4.6x10 ⁶	4.6x10 ⁶	4.6x10 ⁶	4.6x10 ⁶	4.6x10 ⁶	4.6x10 ⁶	4.6x10 ⁶	4.6x10 ⁶	4.6x10 ⁶
18 h	0	1.8x10 ⁶	4.6x10 ⁵	5.0x10 ⁵	1.0x10 ²	1.9x10 ⁶	1.0x10 ⁵	2.1x10 ⁵	1.1x10 ³	2.0x10 ⁶	1.3x10 ⁶	2.6x10 ⁶

Table 7. Photocatalytic inactivation of *C. albicans* in phosphate solution at various pHs

	Phosphate 4.17		Phosphate 6.08		Phosphate 7.1		Phosphate 8.42	
Initial pH	4.47		6.06		7.07		7.90	
	L	D	L	D	L	D	L	D
Final pH	4.12	4.44	6.22	6.13	7.12	7.11	7.89	7.96
0 h	2.1×10^7	2.1×10^7	2.1×10^7	2.1×10^7	2.1×10^7	2.1×10^7	2.1×10^7	2.1×10^7
20 h	4.1×10^6	2.1×10^6	5.1×10^6	1.0×10^7	1.5×10^6	2.7×10^6	5.5×10^5	3.2×10^6

To distinguish if the bicarbonate/carbonate ions or the change in H^+ and OH^- concentration as a function of pH is the basis of the changes inactivation activity, photocatalytic inactivation tests were performed with water at pH 6.00, 8.05 and 10.00. The data in Tables 8, 9, and 10 clearly show that bicarbonate ion is necessary to improve the photocatalytic antifungal properties of P25.

Table 8. Photocatalytic inactivation of *C. albicans* with P25 in water at an initial pH of 6.00

	TiO ₂ (1.0 mg/mL)				Water	
	Light	Light	Dark	Dark	Light	Dark
Final pH	6.08	6.91	5.97	6.01	6.51	5.58
0 h	7.5×10^6	7.5×10^6	7.5×10^6	7.5×10^6	7.5×10^6	7.5×10^6
24 h	2.6×10^6	7.7×10^6	3.4×10^6	2.1×10^6	2.85×10^6	2.36×10^6

Table 9. Photocatalytic inactivation of *C. albicans* with P25 in water at an initial pH of 8.5

	Water pH (8.5)					
	TiO ₂ (1.0 mg/mL)				Control	
	Light	Light	Dark	Dark	Light	Dark
Final pH	5.79	5.45	5.52	5.35	7.07	5.60
0 h	7.5×10^6	7.5×10^6	7.5×10^6	7.5×10^6	7.5×10^6	7.5×10^6
24 hr	1.0×10^7	2.05×10^7	8.7×10^6	4.0×10^6	5.6×10^7	2.4×10^6

Table 10. Photocatalytic inactivation of *C. albicans* with P25 in water at an initial pH of 10.00

	Water pH (10.00)					
	TiO ₂ (1.0 mg/mL)				Control	
	Light	Light	Dark	Dark	Light	Dark
Final pH	7.18	6.84	7.80	8.45	8.47	6.73
0 h	7.5×10^6	7.5×10^6	7.5×10^6	7.5×10^6	7.5×10^6	7.5×10^6
24 hrs	7.2×10^6	5.3×10^6	4×10^6	3.8×10^6	1×10^5	2.8×10^6

The rate of photocatalytic inactivation *C. albicans* with P25 in sodium bicarbonate buffer was examined at pH 6.00 and pH 10.12 as shown in Figures 2 and 3. No antifungal activity was observed in the first 10-15 h; however, a significant decrease in fungal colonies was observed after 15 h of photolysis in both buffers.

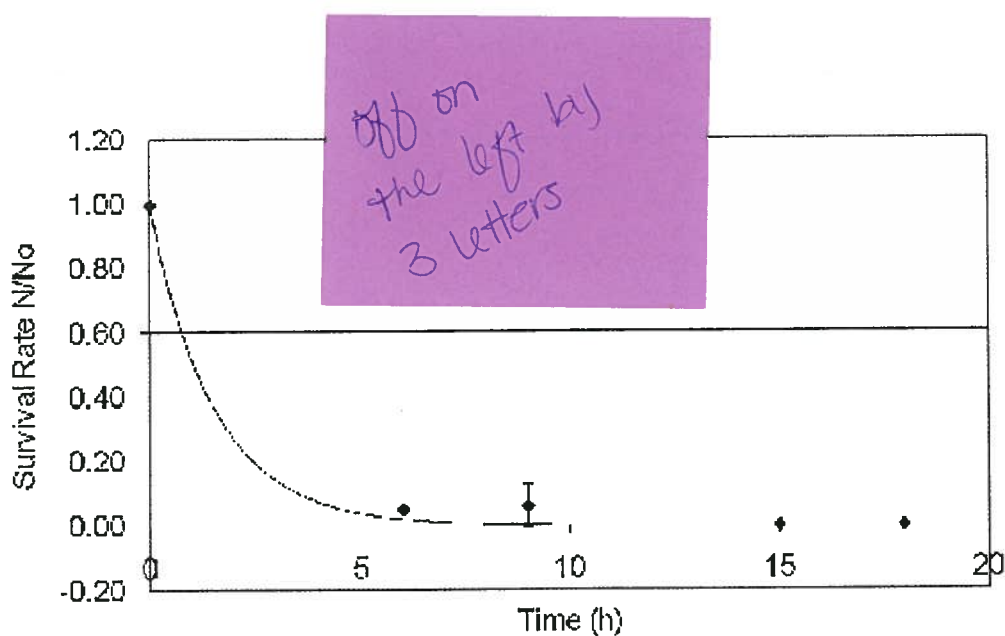


Figure 2. Survival ratio N/N_0 for *C. albicans* under photocatalytic treatment with P25 at pH 6 with a 100 mM bicarbonate solution.

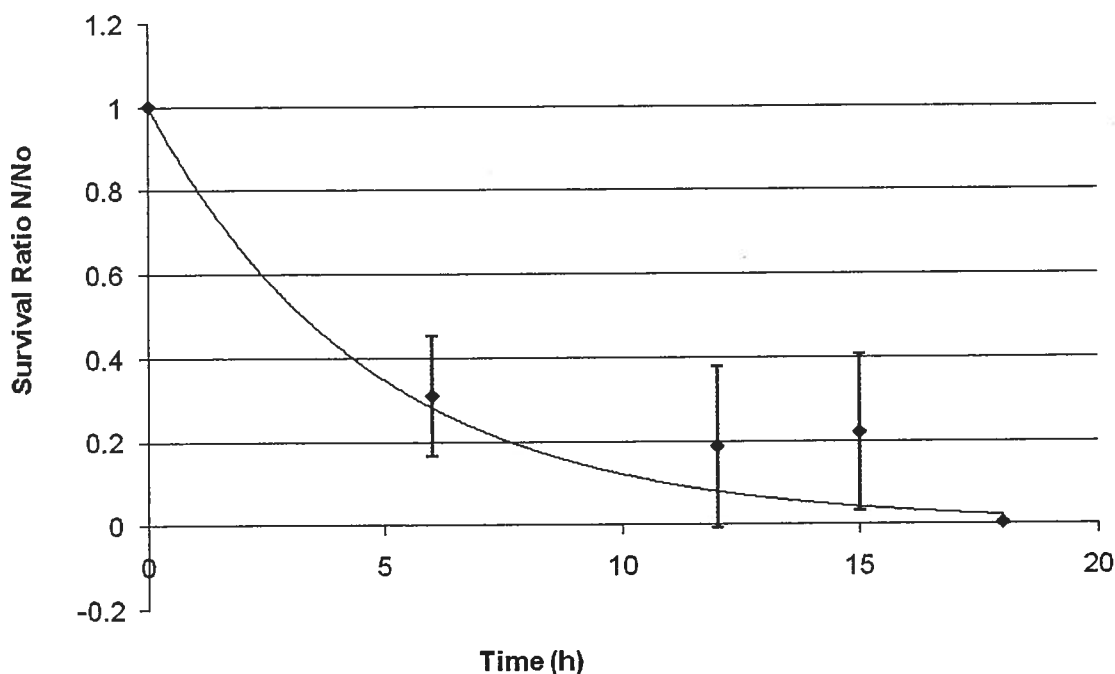


Figure 3. Survival ratio N/N_0 for *C. albicans* under photocatalytic treatment with P25 at pH 10.12 with a 100 mM bicarbonate/carbonate solution.

The change in morphology of *C. albicans* was examined as a function of photocatalytic treatment using an optical microscope at 30x magnification. Panel A in Figure 4 shows *C. albicans* after treatment with bicarbonate buffer in the dark without TiO_2 ; the cells remain round and intact. Panel B shows the *C. albicans* after treatment with warm white light without TiO_2 . These cells look similar to those in section A and show no indication of damage. Panel C shows the *C. albicans* after treatment with sodium bicarbonate and TiO_2 in the dark. The cells in this figure appear to be bound to the surface of the TiO_2 , yet still appear rigid and intact. Panel D shows the *C. albicans* after treatment with TiO_2 under warm white light with sodium bicarbonate. It is observed that the cells are totally fragmented and it appears that most of and the cytoplasm has leaked out.

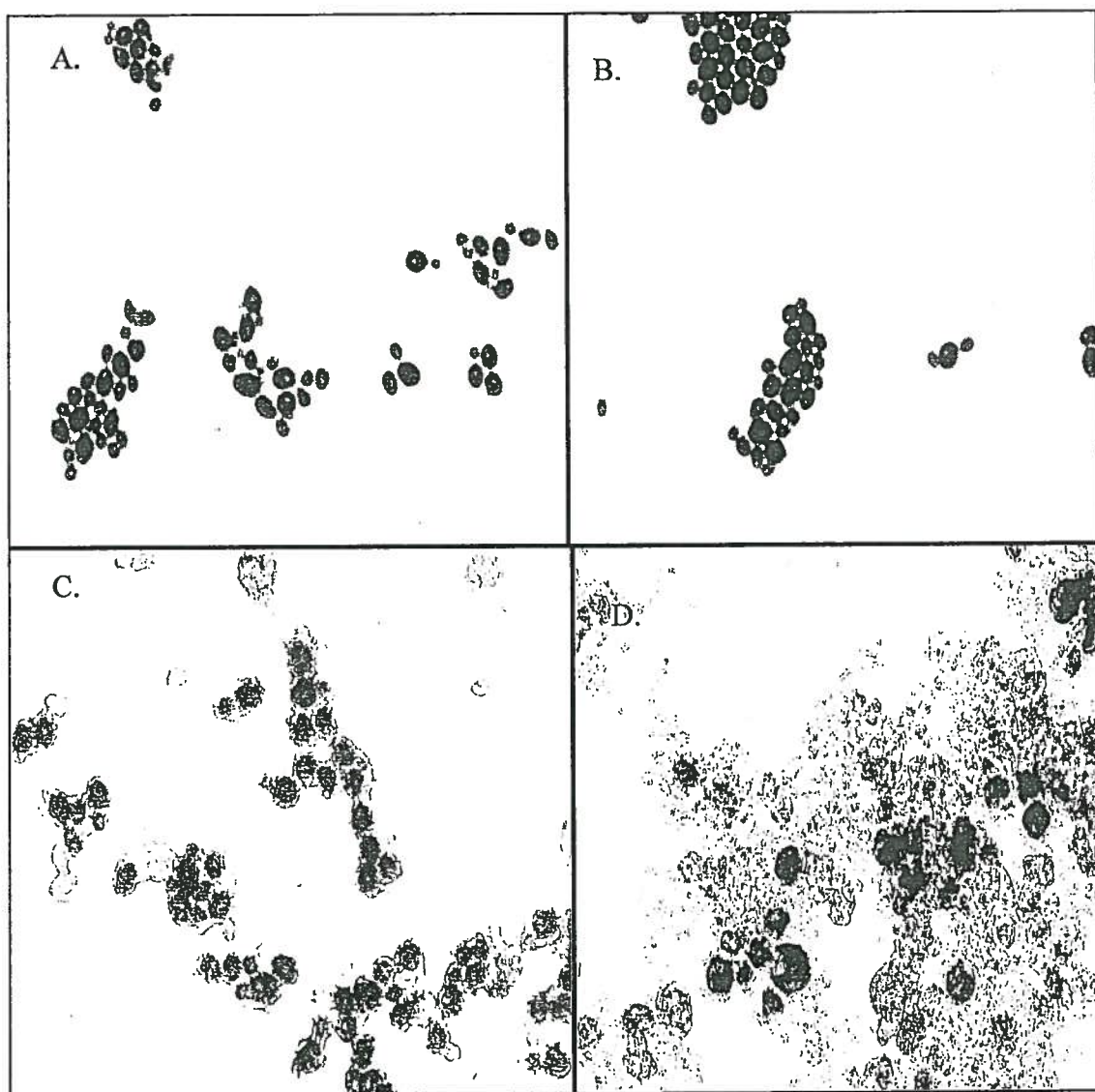


Figure 4. Photomicrographs (30x) of *C. albicans*; A. Dark without TiO_2 , B. Light without TiO_2 , C. Test Dark with TiO_2 , D. Test Light with TiO_2

To detect protein oxidation as a result of photocatalytic antifungal activity of TiO_2 a ketone assay was performed using 2,4-dinitrophenylhydrazine. This test detects ketones and aldehydes generated as a result of oxidative stress caused by photocatalytic activity of TiO_2 on *C. albicans*. There was a significant increase in the 2,4-DNPH reactive

material formed with TiO_2 under the warm white light as compared to in the dark. This data indicates that the inactivation of the fungi occurs with considerable protein oxidation, indicative of oxidative stress, see Figure 5.

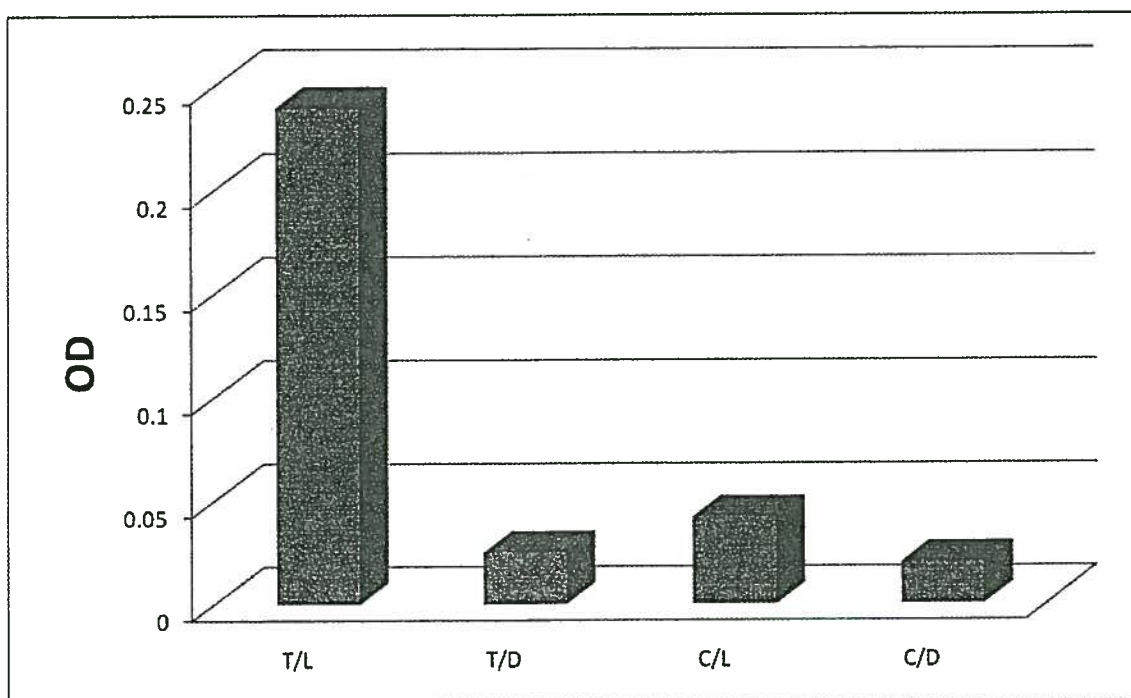


Figure 5. 2,4 DNPH test on *C. albicans* [T/L 1mg/mL of TiO_2 under warm white light], [T/D 1.0 mg/mL of TiO_2 in the dark], [C/L *C. albicans* in pure water under warm white light], and [C/D *C. albicans* in pure water in the dark]

CHAPTER 4

CONCLUSION

Degussa P25 (P25), a mixed phase TiO_2 material (25 % rutile and 75 % anatase), that has been found to be successful for the inactivation of bacteria and viruses was found to be ineffective for the photocatalytic inactivation of fungi in water under similar experimental conditions. Fungi are eukaryotic organisms with cell walls that are composed of polysaccharide polymers, like chitin, β -glucans, mannans and glycoproteins that are much thicker and stronger than the cell walls of bacteria and the capsid of viruses.

The addition of chloride or phosphate to P25 suspensions did not improve the photocatalytic inactivation of the fungi. Addition of alumina led to a slight improvement in photocatalytic inactivation and silver/P25 inactivated the fungi even in the dark. The addition of bicarbonate, which is found in natural waters, dramatically increased the photocatalytic fungi inactivation activity of P25. The photocatalytic inactivation activity of P25 in bicarbonate was found to be pH dependent, with activity increasing with decreasing pH, indicating that HCO_3^- is more active than CO_3^{2-} in the inactivation of the fungi. Based on microscopic examination of the morphology of *C. albicans* upon photocatalytic inactivation with P25 and carbonate it was found that the

cell walls are seriously comprised and cytoplasm leaked out during photocatalytic treatment.

To understand the role of the bicarbonate ion on the photocatalytic inactivation of fungi with P25 model systems should be examined. For example, the photocatalytic activity P25 and P25/bicarbonate with polysaccharide polymers, β -glucans, mannans and glycoproteins should be examined to determine the role of the bicarbonate ion. This may allow more effective inactivation conditions to be determined.

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49. Dr. Liang Liao provide the silver doped $\text{TiO}_2/\text{Al}_2\text{O}_3$ composite prepared by the sol-gel method using HIO_3 as a catalysts, manuscript in preparation.